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**14. ABSTRACT:**
NF1 is a common dominant genetic disorder characterized by multiple benign and malignant tumors of neural origin and, often, cognitive deficits in children. How mutations in the NF1 gene lead to severe learning deficits is largely unknown. The objective of this proposal is to test the hypothesis that the newly identified NF1-Syndecan2-CASK signaling complex plays an essential role in the regulation of synaptic Ras-MAPK activity and dendritic spine maturation. Using several siRNAs and dominant negative constructs for NF1 GAP activity to specifically knockdown or inhibit NF1, we have obtained compelling evidence showing that NF1 deficiency indeed leads to abnormal development of dendritic spines and hyperactive Ras-MAPK activity, and furthermore, these deficits can be rescued by overexpression of NF1 GRD I, a central domain of NF1 responsible for its Ras GAP activity. We have also begun to determine whether NF1-deficient cells have an altered capacity to undergo morphological plasticity after spaced depolarizing stimuli, and whether the deficits in morphology can be rescued by manipulating the Ras-MAPK activity.

**15. SUBJECT TERMS** NF1, SYNDECAN2, CASK, RAS-MAPK SIGANLING, DENDRITIC SPINES, IMMUNOCYTOCHEMISTY, FRET, PLASTICITY, EPHB2, NF1 KNOCKOUT MICE, DENTATE EXPLANT CULTURES, TIMELAPSE-IMAGING, CONFOCAL MICROSCOPY, RNA INTEREFENCE, DOMINANT NEGATIVE MUTANTS

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## I. Introduction:

Neurofibromatosis type 1 (NF1) is one of the most common dominant genetic disorders characterized by multiple benign and malignant tumors of neural origin. About 50% of NF1 children also exhibit cognitive deficits such as spatial learning defects and reading difficulty. How mutations in a single gene lead to severe learning deficits is largely unknown. The protein encoded by NF1, neurofibromin, contains a GAP domain, known to inhibit Ras-mediated signal transduction. A recent report from Silva's group demonstrated that the learning deficits of heterozygous null mutant (Nf1+/-) mice could be rescued by genetic and pharmacological manipulations that decrease Ras function (Costa et al., 2002), suggesting that a tightly regulated Ras activity is critical for its function in synaptic plasticity. NF1 forms a tripartite complex with CASK, a synaptic PDZ protein, and Syndecan 2, a heparan sulfate proteoglycan (HSPG) (Hsueh et al., 2001). CASK has previously been proposed to function as multi-domain scaffolding protein that organizes specific signaling complexes at cell contact sites, and may have a role in receptor localization. HSPGs are believed to function as co-receptors in many receptor tyrosine kinase signaling pathways, and Syndecan 2 is known to promote dendritic spine maturation. Therefore, in principle, this protein complex can function in both organizing the synaptic protein complex and mediating key signal transduction events during synaptogenesis and synaptic plasticity (Fig.1). The objective of this study is to combine structural and functional analyses in conjunction with the assessment of the underlying signal transduction mechanisms at single cell level, to better understand the precise NF1 function in neurons and how deregulation of this function leads to cognitive deficits in NF1 patients.

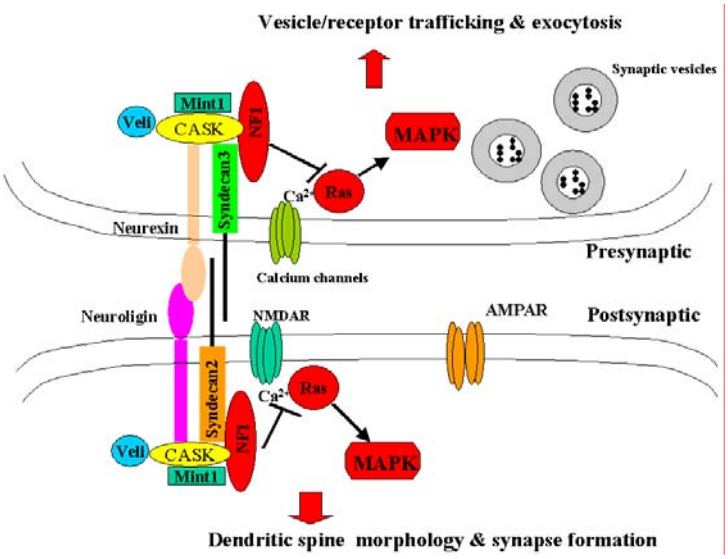


**Fig.1.** ***NF1 may be part of critical signaling networks underlying synapse formation and function.*** Model highlights the protein-protein interaction of NF1 with several key synaptic signaling molecules previously implicated in the differentiation of both presynaptic and postsynaptic structure and function. On the postsynaptic side, Ras-MAPK signaling is involved in the initiation of dendritic filopodia; however, a spatiotemporal shutting-off of its activity by NF1 may be required for stabilization and development of fully functional dendritic spines.

## II. Body:

We propose to use multidisciplinary approaches, including time-lapse imaging confocal microscopy, molecular imaging with FRET, quantitative immunocytochemistry, and genetic mouse models as well as pharmacological and molecular manipulations such as dominant negative constructs and small interfering RNAs (siRNAs), to define the NF1 function in synapse formation and morphogenesis of dendritic spines. The three major tasks of this study are:

**Task1:** To determine if the NF1-syndecan-CASK signaling complex is an upstream regulator of the synaptic Ras-MAP kinase pathway

**Task2:** To assess the role of the NF1-syndecan-CASK signaling complex in regulation of dendritic spine morphology

**Task3:** To determine if NF1-deficient cells or NF1 deficient mice have an altered capacity to undergo

morphological plasticity after spaced depolarizing stimuli, and if deficits in morphology can be rescued by manipulating Ras-MAPK signaling.

During the last funding period, we made several useful siRNAs for specific knockdown of NF1 and two dominant negative constructs to inhibit NF1 GAP activity, and refined the methodology for monitoring Ras activity with FRET probes for Ras. With those tools and reagents, we have now obtained compelling evidence showing that NF1 deficiency indeed leads to abnormal development of dendritic spines and hyperactive Ras-MAPK activity; and furthermore, these deficits can be rescued by overexpression of NF1 GRD I, a central domain of NF1 containing ~360 residues responsible for its Ras GAP activity. In the following sections, I will highlight these exciting new findings in more detail.

**1). Task1:** To determine if NF1-syndecan-CASK signaling complex is an upstream regulator of the synaptic Ras-MAP kinase pathway

During the last funding period, using immunoblot analysis and immunohistochemistry, we demonstrated that, as seen in many other types of cells, Nf1+/- neurons displayed hyperactive basal and membrane depolarization evoked MAPK activity as compared to wild type neuron. In parallel, we showed that specific knockdown of NF1 protein produced hyperactive pMAPK in Hela cell lines and cultured hippocampal neuron. We have now obtained evidence that Nf1+/- neurons had higher Ras GTPase activity by GST pull-down experiment (Fig.2). To monitor the spatiotemporal activation of Ras in real time, we have made use of a fluorescence resonance energy transfer (FRET)-based probe for Ras. To specifically ask if NF1 regulates synaptic Ras activity, we have taken advantage of the regional photobleaching tools of the confocal system. We have selectively photobleached single spines to measure Ras activity at the single synapse level, and our preliminary data showed that Ras is indeed activated by neuronal activity at dendritic spines, and Nf1+/- neurons have elevated basal Ras activity both in soma and spines.

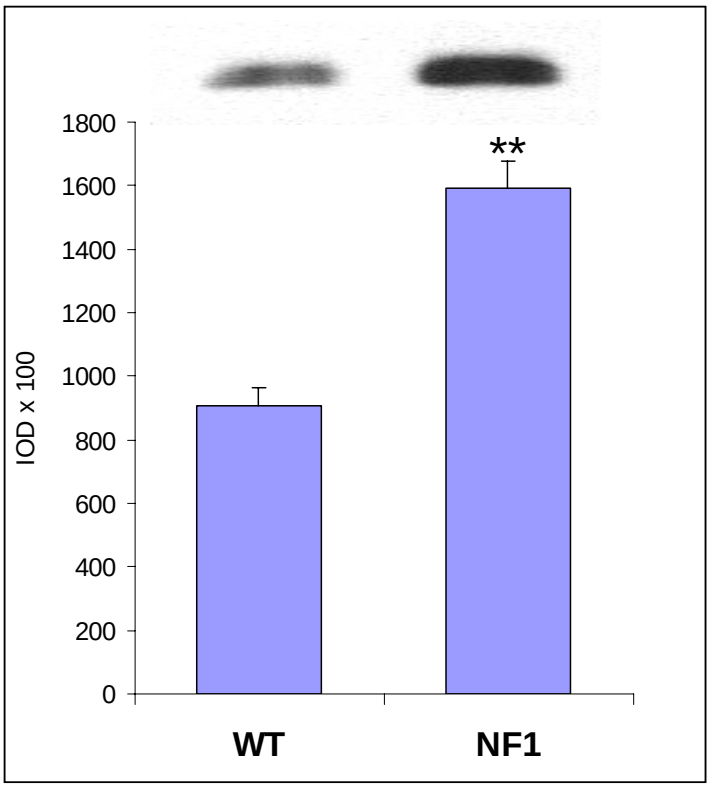


**Fig.2. Hyperactive Ras in Nf1+/- animals.** Ras activity was measured using a kit obtained from Upstate Biotechnology. Hippocampus was dissected from 1 month old wild type (WT) and NF1 (+/-) brains (n=3), dounce homogenized in hypotonic lysis buffer, and 500 μg aliquots were diluted in assay buffer along with Raf-1-agarose beads to pull down active p21 Ras. Beads were washed, resuspended in SDS sample buffer, and resolved by SDS-PAGE. Western blots were probed with an antibody to p21 Ras, and densitometric values were obtained. **$p<0.01$, Student's T-test.

Growing evidence suggests that Ras activates multiple parallel downstream pathways, besides the Ras-MAPK pathway, to mediate its function. Several groups recently showed that Ras-Akt-mTOR signaling (Altomare and Testa, 2005; Dasgupta et al., 2005; Johannessen et al., 2005; Yin et al., 2005) is

altered in NF1 disease models. We and Morgan Sheng's group recently independently demonstrated that the Ras-Akt-mTOR signaling pathway plays a central role in the regulation of dendrite size and shape as well as dendritic spine morpgology (Jaworski et al., 2005; Kumar et al., 2005). Interestingly, genetic defects of the Ras-PI3K-AKT-mTOR signaling pathway, either through genetic inheritance or as a spontaneous genetic mutation, are also associated with other human diseases such as tuberous sclerosis (TSC). TSC1/2 is known to act downstream of AKT to negatively regulate mTOR. Remarkably, Bernardo Sabatini's group recently showed that TSC1/2 also plays important role in regulation dendritic morphology (Tavazoie et al., 2005). Taken together, these new findings raise the interesting possibility that a common cause of the neurological symptoms seen in these diseases may be linked to abnormal dendrite development. Future work to further investigate a possible involvement of the Ras-Akt-mTOR signaling in NF1 and its role in the cognitive deficits in patients would be of great interest.

**2). Task 2:** To assess the role of the NF1-syndecan-CASK signaling complex in regulation of dendritic spine morphology

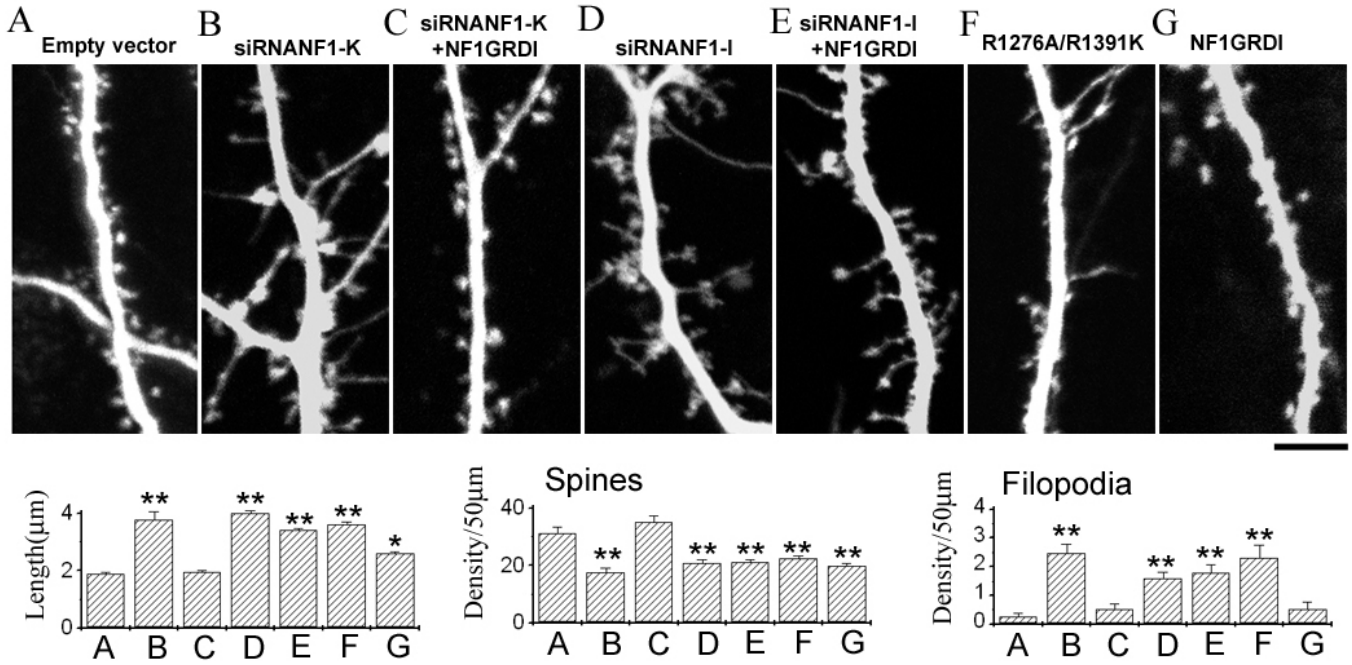


**Fig.3 Overexpression of NF1 siRNAs and dominant negative NF1produced immature spine phenotypes with prominent filopodia or loss of dendritic spines.** DG explants were co-transfected EGFP with different constructs at 7 DIV and imaged at 17DIV. The upper panels show typical spine morphology in the respective groups. The lower panels show quantification for spine and filopodium density, and spine length. Value are means +/-SEM, * $p<0.05$; ** $p<0.01$, one-way ANOVA. Note that co-expression of $siRNA_{NF1-K}$ with NF1GRDI but not co-expression of $siRNA_{NF1-i}$ with NF1GRDI largely rescued the immature spine phenotype. The dominant negative R1276A/R1391K for NF1 GAP also produced a similar immature spine phenotype. Data were from two independent experiments. N = (A: 715 spines, 18 neurons); (B: 599, 28); (C: 353, 11); (D: 319, 32); (E: 424, 25); (F: 501, 30); (G: 472, 19). Scale bar, 10μm.

Our preliminary results in rat hippocampal cultures showed that overexpression of Syndecan2 promoted the maturation of dendritic spines; overexpression of CASK, on the other hand, seemed to promote the maturation of both the presynaptic boutons and dendritic spines in immature neurons. We have continued making excellent progress showing that knockdown of each of the three proteins using a vector-based RNA mediated interference (RNAi) method produced a similar immature spine phenotype with prominent filopodia or loss of dendritic spines in mature neurons (Fig.3). We have now obtained compelling evidence showing that specific knockdown of NF1 leads to abnormal dendritic spine morphology. We have been focusing on two of the siRNAs that target regions in the NF1GRDI

($siRNA_{NF1-I}$) or outside ($siRNA_{NF1-K}$) of the NF1GRDI, respectively. Both constructs significantly reduced the level of NF1 to a similar level (more than 50%) when tested in HeLa cells; and as expected, pMAPK activity was significantly increased in cells expressing both constructs. Remarkably, overexpression of NF1GRDI was able to largely rescue the pMAPK level in cells co-expressing $siRNA_{NF1-K}$ with NF1GRDI. Cells co-expressing $siRNA_{NF1-i}$ with NF1GRDI still showed elevated hyperactive pMAPK and the slightly reduced pMAPK activity was due to incomplete knockdown of NF1GRDI by $siRNA_{NF1-I}$. Our latest experiments showed that co-expression of $siRNA_{NF1-K}$ with NF1GRDI but not co-expression of $siRNA_{NF1-I}$ with NF1GRDI largely rescued the immature spine phenotype (Fig.3), strongly suggesting that these observed immature phenotypes indeed were due to loss of NF1 function. We are now directly correlating the morphological changes with the extent of the relative hyperactive Ras-MAPK activity in these neurons.

It has been shown that overexpression of dominant negative constructs for NF1 GAP activity inhibits outgrowth of neurite in PC12 cells and in early stages of development in hippocampal primary cultures. Using one of the dominant negative constructs for NF1 GAP (R1276A/R1391K), we showed that, similar to the siRNAs for NF1, R1276A/R1391K also produced an immature spine phenotype and reduced spine density (Fig. 3).

To further demonstrate that the tripartite NF1-syndecan-CASK signaling complex plays a key role in spine maturation, we have begun to co-transfect Syndecan2 or CASK with siRNAs for NF1 or the dominant negative NF1GAP constructs, and our preliminary data showed that knockdown of NF1 blocked the effect of CASK on spine maturation, suggesting that down-regulation of Ras activity by recruiting NF1 to the CASK-Syndecan2 signaling complex likely plays an essential role in stabilization and maturation of dendritic spines.

**3). Task3:** To determine if NF1-deficient cells or NF1 deficient mice have an altered capacity to undergo morphological plasticity after spaced depolarizing stimuli, and if deficits in morphology can be rescued by manipulating Ras-MAPK signaling.

This aim will complement the studies in Aim1 & 2 on the role of NF1 in development, and determine if NF1 also plays an essential role in dendritic spine plasticity. If so, additional pharmacological interventions and genetic manipulations of the Ras-MAPK pathway will be used to rescue the morphological deficits. A similar combination of the confocal time-lapse imaging of morphological changes with the assessment of spatiotemporal activation of the Ras-MAPK pathway will further link the morphological plasticity to changes in signal transduction pathways in this activity-dependent model. We have successfully established organotypic slice culture method in the lab. In a separate study, we have used slice culture system to demonstrate a central role of the Ras-PI3K-mTOR signaling pathway in the regulation of dendrite size and shape (Kumar et al., 2005). We have begun to investigate a possible role of NF1 in activity-dependent dendritic spine remodeling using both DG-CA3 explants and CA1/3 slice cultures. We will complete all the proposed experiments as originally planned. If we are able to make good progress ahead of the schedule, we will proceed to express the NF1GRD as well as manipulate Ras-AKt-mTOR signaling to try to rescue the abnormalities of dendritic spine development and plasticity.

**III. Key Research Accomplishments:**

· Refined the FRET probe for Ras and used GST pull-down assay to confirm that Nf1+/- neurons or cells with knockdown of NF1 display hyperactive basal Ras activity; using immunoblot analysis and immunohistochemistry demonstrated that Nf1 deficiency leads to hyperactive basal and evoked MAPK activity.

· Obtained compelling evidence showing that specific knockdown of NF1 produced hyperactive Ras-MAPK signaling and immature spine phenotype; and these deficits can be rescued by overexpression of NF1 GRD I, a central domain of NF1 containing ~360 residues responsible for its Ras GAP activity.

· Developed two dominant negative constructs for NF1 GAP activity and their effects on Ras-MAPK activity and spine morphology are being tested.

· Demonstrated that overexpression of CASK promoted the maturation of both the presynaptic boutons and dendritic spines with concomitant reduction of MAPK activity in immature neurons; obtained preliminary data showing that NF1 is required for mediating the effect of CASK on spine maturation.

· Established mouse DG explants and CA1/3 organotypic cultures; and have begun to investigate a possible role of NF1 in activity-dependent dendritic spine remodeling.

**IV. Reportable outcomes:**

**1). Papers**

1. Kumar, V., Zhang, M.X., Swank, M.W., Kunz, J. and Wu, G-Y. (2005) Regulation of Dendritic Morphogenesis by Ras-PI3K-Akt-mTOR and Ras-MAPK Signaling Pathways, J. Neurosci. 25: 11288-11299.
2. Ryan, X.P., Alldritt, J.L., Allen, P., Wu, G-Y*, Nairn, A.C.* and Greengard, P. (2005) The rho-specific GEF, Lfc, interacts with neurabin and spinophilin to regulate dendritic spine morphology, Neuron, 47:85-100 (* Co-corresponding authors).
3. Swank, M.W., Kumar, V. and Wu, G-Y. A novel method of loading samples onto mini-gels for SDS-PAGE: Increased sensitivity and western blots using sub-microgram quantities of protein, J. Neurosci. Methods, in revision.
4. Bryan, B., Cai, Y., Wrighton, K., Wu G-Y, Feng, XH and Liu, M. (2005) Smurf1 promotes dendritic outgrowth and dendritic spine morphorgensis via ubiquitination of RhoA, FEBS Letters, 579:1015-9.
5. Maria V. Tejada-Simon, M.V., Serrano, F., Villasana, L.E., Kanterewicz, B.I., Wu, G-Y, Quinn M. and Klann, E., Synaptic localization of a functional NADPH oxidase in the mouse hippocampus, MCN, 29:97-106.
6. Wu, G.Y. and Wang, S.R. Telecephalic Striatum Exerts Inhibitory Action on Binocular Neurons in the Toad's Tegmentum, Neuroscience. Letters, submitted.
7. Alldritt, J.L., Ryan, X.P., Allen, P., Nairn, A.C. Greengard, P. and Wu, G-Y, Lfc Regulates Dendrite Formation through Rho-ROCK signaling pathway, J. Neurosci., in preparation.
8. Kumar, V., Zhang, M.X., Cao Y-Q, Chen G., Tsien, R.W. and Wu, G-Y, Rapidly reversible dendritic swelling is correlated with protein mobilization and formation of dendritic protrusions, PNAS, in preparation.
9. Zhang, M.X., Kumar, V., Chen, G., Cao Y-Q, Deisseroth, K., Tsien, R. W. and Wu, G-Y, Expression of an active Ras alters the maturation of dendritic spines, in preparation.
10. Long, C., Kumar, V., Yuan, L., and Wu, G-Y, U0126, a MEK inhibitor reduces potassium current and evokes synchronized firing in cultured hippocampal neurons, in preparation.

**2). Presentations:** Annual Neuroscience Meeting, Washington D.C., 2005

1. Kumar V., Levesen JM, M. Swank M., JD Sweatt JD and Wu, G.Y. Regulation of Brain Size and Dendrite Morphology by mTOR Signaling in vivo, Soc. Neurosci. Abstr. 2005, 25.8.

2. Long, C., Kumar V., M. Swank M. and Wu, G.Y. Akt Differentially Regulates Synaptic Growth and Strength Through mTOR-Dependent and -Independent Mechanism, Soc. Neurosci. Abstr. 2005, 25.9.

**3). Patents**

1. Swank M.W. and Wu, G-Y, pending US patent #11/248401 "Micro-loading Device", filed on October 12, 2005

**4). Grants**

"Cell Signaling and Dendritic Spine Plasticity" 2/01/2006-11/30/2010
Type: R01 (1R01NS055339-01A2) Agency:NIH/NINDS $262,500
The major goals of this project are to determine whether NF1 plays an essential role in synapse formation and dendritic spine plasticity through its role as a negative regulator for Ras (and MAPK) signaling.

"A central role of the Ras-PI3K-Akt-mTOR pathway in dendritic morphogenesis"
12/01/2005-11/30/2008
Type: Research Grant Agency: Whitehall Foundation $75,000
The goal of this project is to define the central role of the Ras-PI3K-Akt-mTOR pathway in the regulation of dendrite size and shape.

**5). Ph.D. Dissertation**

Jacqueline Lee Alldritt was awarded a Ph.D. on June 10, 2005.

**V. Conclusions**

In summary, the NFRP New Investigator Award has provided us the crucial support for our research on the biological function of the newly identified NF1-syndecan-CASK signaling complex. With the support, we have been able to make significant progress on several innovative approaches to manipulate the signaling complex and image the underlying signal transduction mechanisms at single cell level. With the several siRNAs for NF1 and the two dominant negative constructs for NF1 GAP activity that we generated during last funding period, we have now obtained compelling evidence showing that NF1 deficiency indeed leads to abnormal development of dendritic spines and hyperactive Ras-MAPK activity, and furthermore, these deficits can be rescued by overexpression of NF1 GRD I, a central domain of NF1 containing ~360 residues responsible for its Ras GAP activity. We will continue the proposed study as planned during the third year of the NFRP support. Our novel results that NF1 deficiency leads to abnormal dendritic spine maturation resulting from hyperactive Ras-MAPK signaling would have important implications for developing therapeutic strategies to target the cognitive deficits in NF1.

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Tavazoie, S. F., Alvarez, V. A., Ridenour, D. A., Kwiatkowski, D. J., and Sabatini, B. L. (2005). Regulation of neuronal morphology and function by the tumor suppressors Tsc1 and Tsc2. Nat Neurosci *8*, 1727-1734.

Yin, B., Morgan, K., Hasz, D. E., Mao, Z., and Largaespada, D. A. (2005). Nf1 gene inactivation in acute myeloid leukemia cells confers cytarabine resistance through MAPK and mTOR pathways. Leukemia.

**VII. Appendices:** None